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Identification of *Vibrio parahaemolyticus* Strains at the Species Level by PCR Targeted to the *toxR* Gene

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The DNA colony hybridization test with the polynucleotide probe for *Vibrio parahaemolyticus toxR* gene was performed. All 373 strains of *V. parahaemolyticus* gave positive results, and the strains belonging to four other *Vibrio* species including *Vibrio alginolyticus* gave weakly positive results, suggesting that *toxR* sequence variation may reflect the phylogenetic relationships of *Vibrio* species. We then established a *toxR*-targeted PCR protocol for the specific detection of *V. parahaemolyticus*.

Vibrio parahaemolyticus is a marine bacterium, and some strains can cause gastroenteritis in humans through the consumption of contaminated seafood. Molecular epidemiological studies revealed a strong correlation between the possession of particular hemolysin genes (*tdh*, *trh*, or both) and the ability to cause disease, supporting the fact that these genes are important virulence genes (4, 16). However, a small portion of clinical strains carried neither of the virulence genes (4, 16). Therefore, the isolation of the organism in the clinical setting and from food samples suspected of being the source of infection, followed by identification of the isolated strains, is still the standard procedure in investigations of gastroenteritis due to *V. parahaemolyticus*. If a PCR method allows detection of the nucleotide sequence specific to *V. parahaemolyticus*, it can facilitate identification of the organism.

The genes encoding two hemolysins designated TLH and δ-VPH of *V. parahaemolyticus* were examined by Taniguchi et al. (18, 19). These genes appear to be fairly specific to *V. parahaemolyticus*. Lee et al. (6, 7) cloned a 0.76-kb nucleotide sequence of unknown function and claimed that the nucleotide sequence is specific to *V. parahaemolyticus* by hybridization and PCR methods (6, 7). However, only limited numbers of strains were examined in those studies, and the relationships of the hemolysin genes and the 0.76-kb sequence with the phylogeny of *V. parahaemolyticus* are not known. For identification purposes, it is ideal to use a nucleotide sequence that is well conserved and that reflects the phylogenetic relationship. rRNA sequences are often used for this purpose. However, the rRNA sequence homologies between *V. parahaemolyticus* and related species are so high that the rRNA sequence does not appear to be suitable for the purpose described above. For example, the 16S rRNA sequences of *V. parahaemolyticus* and *Vibrio alginolyticus* are >99% identical (5, 15). The *gyrB* gene encodes the B subunit of DNA gyrase, which is essential for DNA replication. The homology of the *gyrB* sequences between *V. parahaemolyticus* and *V. alginolyticus* is 86.8% (20). For this reason, a PCR procedure targeting the *gyrB* gene was recently developed for the specific detection of *V. parahaemolyticus* in shrimp (20).

The *toxR* gene was first discovered as the regulatory gene of the cholera toxin operon, but it was later shown to be involved in the regulation of many other genes in *Vibrio cholerae* (1, 9). We subsequently found the *toxR* gene in *V. parahaemolyticus* and demonstrated its regulatory function (8). The *toxR* gene sequences have also been cloned from *Vibrio fisheri* and at least two other species of *Vibrio*, and their sequences have been analyzed (13, 14). Therefore, the *toxR* gene appears to be well conserved among *Vibrio* species. The degree of homology of the *toxR* gene between *V. parahaemolyticus* and *V. cholerae* (52% identity) is much lower than that of the rRNA gene (91 to 92% identity) (5, 8). We therefore investigated in this study whether the *toxR* gene sequence can be used to develop a PCR method for the specific identification *V. parahaemolyticus*.

Phenotypic characteristics of bacterial strains. The bacterial strains used in this study are listed in Table 1. Clinical strains listed as *V. parahaemolyticus*, listed as having an unknown identification (non-*V. parahaemolyticus*), and identified as known *Vibrio* species (11 strains; see below) were isolated from patients at hospitals in India, Bangladesh, and Korea and at quarantine stations in Japan during the period between 1977 and 1997 (11, 12). The nonclinical strains were isolated from seawater in Korea in the summer of 1996. The seawater samples were plated, after enrichment in alkaline peptone water, onto thiosulfate-citrate-bile salts-sucrose agar (Eiken Chemical, Co., Ltd., Tokyo, Japan), and blue-green colonies were selected. All clinical and nonclinical strains examined in this study were tested for the characteristics listed in Table 2 by standard procedures (3) except that the NaCl concentration of the test medium was adjusted to 1.5%.

The phenotypic characteristics of these 494 strains were examined in two steps. The following characteristics were examined in the initial screening step. The strains that showed an alkaline top, an acidic bottom, and no H_2S production in the TSI reaction and that gave positive results in the lysine decarboxylase, indole, and motility tests were selected. These strains were further examined for the other characteristics listed in Table 2. Three hundred sixty-five strains that gave the results listed in Table 2 were identified as *V. parahaemolyticus*. Seven, two, two, and one strain were identified as *Vibrio mimicus*, *Vibrio furnisii*, *V. alginolyticus*, and *V. cholerae* non-O1 and non-O139, respectively, and are included in Table 1 as such. The other strains could not be assigned to the species listed

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TABLE 1. Results of tests for *toxR* gene

Species	Source or strain no.[a]	Detection of *toxR* gene[b] by the following assay:	
		DNA probe	PCR
V. parahaemolyticus	C	+ (363/363)	+ (363/363)
V. parahaemolyticus	N	+ (10/10)	+ (10/10)
V. cholerae O1	C	− (12/12)	− (12/12)
V. cholerae O139	C	−	−
V. cholerae non-O1, non-O139	C	− (11/11)	− (11/11)
V. mimicus	C	− (18/18)	− (18/18)
V. fluvialis	C	− (12/12)	− (12/12)
V. hollisae	C	− (7/7)	− (7/7)
V. vulnificus	C	− (7/7)	− (7/7)[c]
V. vulnificus	N	− (4/4)	− (4/4)[d]
V. furnisii	C	− (2/2)	− (2/2)
V. furnisii	N	−	−
V. alginolyticus	N	+W (10/10)	− (10/10)
V. aestiarianus	ATCC 35048	−	−
V. anguillarum	PT87050	−	−
V. campbellii	ATCC 25920	+W	−
V. carchariae	ATCC 35084	+W	−
V. cincinnatiensis	ATCC 35912	−	−
V. damsela	ATCC 33539	−	−
V. diazotrophicus	ATCC 33466	−	−
V. gazogenes	ATCC 29988	−	−
V. harveyi	ATCC 14126	+W	−
V. ichthyoenteri	IFO15847	−	−
V. iliopiscarius	ATCC 51760	−	−
V. logei	ATCC 15382	−	−
V. mediterranei	ATCC 43341	−	−
V. metschnikovii	RIMD2208006	−	−
V. mytili	ATCC 51288	−	−
V. nereis	ATCC 25917	−	−
V. navarrensis	ATCC 51183	−	−
V. nigripulchritudo	ATCC 27043	−	−
V. ordalii	ATCC 33509	−	−
V. orientalis	ATCC 33934	−	−
V. mediterranei	ATCC 43341	−	−
V. pelagius	ATCC 25916	−	−
V. penaeicida	IFO15640	−	−
V. proteolyticus	NCMB1326	−	−
V. splendisdus	ATCC 33125	−	−
V. tubiashii	ATCC 19109	−	−
Aeromonas hydrophila	C	− (10/10)	− (10/10)
Aeromonas sobria	C	− (10/10)	− (10/10)
Plesiomonas shigelloides	C	− (10/10)	− (10/10)
Escherichia coli	C[e]	− (20/20)	− (20/20)
Shigella dysenteriae	C	− (2/2)	− (2/2)
Shigella boydii	C	− (2/2)	− (2/2)
Shigella sonnei	C	− (3/3)	− (3/3)
Salmonella choleraesuis	C[f]	− (10/10)	− (10/10)
Staphylococcus aureus	C[g]	− (4/4)	− (4/4)
Unknown[h]	C	− (22/22)	− (22/22)
	N	− (86/86)	− (86/86)

[a] C, clinical (human); N, nonclinical (environmental).
[b] +, detected; +W, weak reaction detected; −, not detected. The values in parentheses indicate the number of positive strains/number of strains tested for multiple strains.
[c] Three strains produced amplicons larger than the specific amplicons.
[d] Two strains produced amplicons larger than the specific amplicons.
[e] Included enteropathogenic (7 strains), enteroinvasive (3 strains), enterotoxigenic (3 strains), enterohemorrhagic (6 strains), and enteroaggregative (1 strain) types.
[f] Included six different O serovars.
[g] The *S. aureus* strains produced enterotoxin type A (2 strains) and type B (2 strains).
[h] Explained in the text.

above from the characteristics examined. These strains were not characterized further and are thus included in Table 1 as strains with unknown identifications.

The test strains that were not selected in the initial screening step were not characterized further and, except for eight strains, are included in Table 1 with the strains with unknown identifications. The eight exceptional strains were negative by the lysine decarboxylase test (two strains) or the indole test (six strains). However, these strains carried the *toxR* gene (described below), and therefore, the other characteristics of these strains were examined. These strains had the characteristics listed in Table 2 and carried the *V. parahaemolyticus gyrB* gene (described below). Therefore, these strains were identified as *V. parahaemolyticus* and are included in Table 1 as such.

TABLE 2. Characteristics of the 373 strains of *V. parahaemolyticus* identified

Test	Result[a]	Test	Result
TSI	K/A	Voges-Proskauer	−
H_2S (on TSI)	−	Simmons citrate	+ (98.7)
Lysine decarboxylase	+ (99.5)	Oxidation-fermentation	F
Indole	+ (99.4)	Acid from:	
		Mannitol	+
Motility	+	Inositol	−
		Glucose	+
Growth in:		Arabinose	+ (72.2)
0% NaCl	−	Lactose	−
3% NaCl	+	Maltose	+
7% NaCl	+	Rhamnose	− (95.4)
10% NaCl	−	Sucrose	−
		Mannose	+
Oxidase	+		
Arginine dihydrolase	−		
Ornithine decarboxylase	+ (98.1)		
Methyl red	+ (96.8)		

[a] K/A, alkaline top and acidic bottom; +, positive; −, negative; F, fermentative. If one or more strains gave a variable result, the percentage of the strains that gave the result indicated by the sign is given in the parentheses.

Most of the strains belonging to other *Vibrio* species and other genera were our laboratory stock strains or strains supplied by other workers for this study, and their phenotypic characteristics were not examined.

DNA colony hybridization. We examined 373 strains of *V. parahaemolyticus* and 290 strains belonging to non-*V. parahaemolyticus* species by the DNA colony hybridization test with the 678-bp *V. parahaemolyticus toxR* gene probe as described previously (8). Marine Agar 2216 (Difco Laboratories, Detroit, Mich.) and an incubation temperature of 25°C were used in place of Luria-Bertani (LB) agar and an incubation temperature of 37°C to grow the standard strains belonging to 26 species of the genus *Vibrio* (those with specific strain numbers in Table 1). The results are summarized in Table 1. All clinical and nonclinical strains of *V. parahaemolyticus* gave clearly positive results. Of the non-*V. parahaemolyticus* strains, those belonging to four *Vibrio* species including *V. alginolyticus* gave positive results, although the hybridization signals were weaker than those for the *V. parahaemolyticus* strains. The other non-*V. parahaemolyticus* strains yielded negative results (Table 1). The hybridization signals of representative strains of *Vibrio* species (reference strains; Table 3) are presented in Fig. 1. The results indicate that the *V. parahaemolyticus toxR* sequence is perfectly conserved among *V. parahaemolyticus* strains and that some other *Vibrio* species carry nucleotide sequences that are fairly homologous to that of the *V. parahaemolyticus toxR* gene. These non-*V. parahaemolyticus* species are phylogenetically closely related to *V. parahaemolyticus* (5, 15).

TABLE 3. Reference strains of *Vibrio* species used in this study

Reference no.	Species	Strain[a]
1	*V. parahaemolyticus*	AQ3855 (O6:K18, *tdh*$^+$, *trh1*$^+$, *trh2*$^-$)
2	*V. parahaemolyticus*	VP-170 (O3:K6, *tdh*$^+$, *trh1*$^-$, *trh2*$^-$)
3	*V. parahaemolyticus*	AQ4537 (O3:K72, *tdh*$^+$, *trh1*$^+$, *trh2*$^-$)
4	*V. parahaemolyticus*	AQ4781 (O1:KUT, *tdh*$^+$, *trh1*$^-$, *trh2*$^+$)
5	*V. parahaemolyticus*	AQ4889 (O4:K12, *tdh*$^+$, *trh1*$^+$, *trh2*$^-$)
6	*V. parahaemolyticus*	KX-V231 (O3:K6, *tdh*$^+$, *trh1*$^-$, *trh2*$^-$)
7	*V. parahaemolyticus*	K-16 (O10:K71, *tdh*$^+$, *trh1*$^+$, *trh2*$^-$)
8	*V. parahaemolyticus*	K-102 (O2:KUT, *tdh*$^-$, *trh1*$^-$, *trh2*$^-$)
9	*V. parahaemolyticus*	VP3 (O2:K3, *tdh*$^+$, *trh1*$^-$, *trh2*$^-$)
10	*V. parahaemolyticus*	VP87 (O1:K1, *tdh*$^+$, *trh1*$^-$, *trh2*$^+$)
11	*V. parahaemolyticus*	VP121 (O3:K6, *tdh*$^+$, *trh1*$^-$, *trh2*$^-$)
12	*V. parahaemolyticus*	W-9802 (O4:K8, *tdh*$^+$, *trh1*$^-$, *trh2*$^-$)
13	*V. parahaemolyticus*	Y-27669 (O8:K22, *tdh*$^+$, *trh1*$^-$, *trh2*$^-$)
14	*V. parahaemolyticus*	AM-8626 (O3:K6, *tdh*$^+$, *trh1*$^-$, *trh2*$^-$)
15	*V. cholerae* O1	NIH41
16	*V. cholerae* O1	NIH35A3
17	*V. cholerae* O139	MO45
18	*V. cholerae* non-O1, non-O139	AM2
19	*V. hollisae*	525-82
20	*V. anguillarum*	PT-87050
21	*V. alginolyticus*	219
22	*V. alginolyticus*	220
23	*V. mimicus*	RIMD2218002
24	*V. vulnificus*	RIMD2219022
25	*V. fluvialis*	RIMD2220002
26	*V. furnisii*	RIMD2223001
27	*V. damsela*	RIMD2222001
28	*V. metchnikovii*	RIMD2208006

[a] The O:K serotype (KUT:K untypeable) and presence (+) or absence (−) of the *tdh*, *trh1*, and *trh2* genes are indicated in parentheses for the *V. parahaemolyticus* strains.

The presence or absence of the *tdh*, *trh1*, and *trh2* genes in the test strain was also examined by the DNA colony hybridization test with polynucleotide probes as described previously (10). Of the 363 clinical strains of *V. parahaemolyticus*, 14 strains (4%) carried neither the *tdh*, *trh1*, nor *trh2* gene. These results encouraged us to develop a PCR protocol that allows the specific detection of the *V. parahaemolyticus toxR* sequence.

PCR. To develop a PCR method specific for the *V. parahaemolyticus toxR* gene, various oligonucleotide primer sets were tested with the reference strains. These strains included 14 strains of *V. parahaemolyticus* and 14 strains of non-*V. parahaemolyticus* species (Table 3). The primer sequences were selected from the regions not conserved between the *V. parahaemolyticus toxR* and the *V. cholerae toxR* sequences (8). Five forward primers and two reverse primers (data not shown) were selected, and all 10 combinations of these primers were tested. The dilution of the boiled culture supernatant, the annealing temperature, and the numbers of amplification cycles of the PCR were varied and the results were compared. Amplicons of the expected sizes were also detected in strains of *V. alginolyticus* and *V. vulnificus* when various PCR primer sets were tested with the reference strains under low annealing temperatures (data not shown). These results supported our hypothesis that the *toxR* gene is well conserved in *Vibrio* species and that variation in the *toxR* sequences may reflect the phylogenetic relationship of *Vibrio* species.

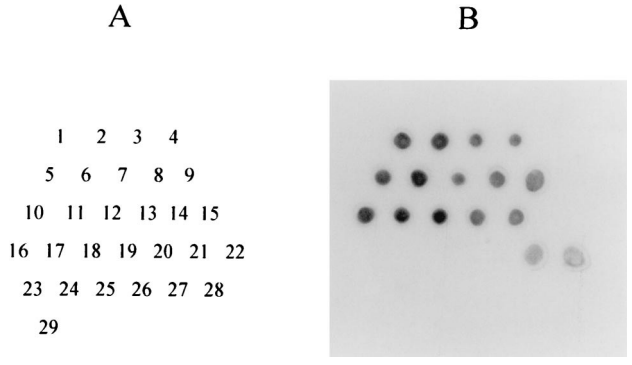

FIG. 1. DNA colony hybridization test with the *V. parahaemolyticus toxR* gene probe for reference strains of *Vibrio* species. (A) Location of the inoculated strains. The numbers correspond to those of the reference strains listed in Table 3. Strain 24 was *Escherichia coli* HB101 (a negative control). (B) Hybridization signals of the test strains detected on X-ray film.

After extensive efforts to optimize the PCR conditions, the following method was shown to allow the specific detection of the *V. parahaemolyticus toxR* gene. The test strain was grown in LB broth containing 1% NaCl at 37°C with shaking (160 rpm) overnight. One milliliter of the culture was boiled for 5 min, and the supernatant was obtained by centrifugation (13,000 rpm) on a tabletop centrifuge (Centrifuge 5415C; Eppendorf, Hamburg, Germany) at room temperature. The supernatant was diluted 10-fold in distilled water. The PCR conditions were as follows. The reaction mixture consisted of 3 μl of the template (supernatant of the boiled culture diluted 1:10), 5 μl of 10× buffer containing 20 mM $MgCl_2$ (Ex Taq buffer; Takara, Tokyo, Japan), 0.25 μl of *Taq* polymerase (Ex Taq; Takara), 4 μl of 2.5 mM deoxynucleoside triphosphate, 2 μl of each primer (10 pmol/μl), and 33.75 μl of distilled water. The amplification conditions were 20 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 63°C for 1.5 min, and extension at 72°C for 1.5 min. The primer sequences were 5′-GTCTTCTGACGCAATCGTTG-3′ (forward) and 5′-ATACGAGTGGTTGCTGTCATG-3′ (reverse). The sequences of the forward and reverse primers correspond to base positions 609 to 629 and 956 to 958, respectively, of the previously described *V. parahaemolyticus toxR* sequence (8). Ten microliters of the reaction mixture was mixed with 2 μl of the dye solution (0.07% bromophenol blue, 20% Ficoll), and the mixture was resolved by electrophoresis in 2% agarose. The 368-bp amplicons of the reference strains obtained by this method are shown in Fig. 2A and B. The culture condition described above allowed enough bacterial growth so that only 20 cycles of amplification was enough to achieve clearly visible amplicon bands.

The PCR protocol established with the reference strains was applied to the examination of all test strains (Table 1). All strains of *V. parahaemolyticus* gave only specific amplicons, whereas non-*V. parahaemolyticus* strains did not. Nonspecific amplicons were generated by only 5 of 11 strains of *V. vulnificus*. These nonspecific amplicons were larger and less evident than the specific amplicons and thus were distinguishable from the specific amplicons (Fig. 2C, lanes 7 to 11). The isolation of *V. vulnificus* from patients with diarrhea appears to be a very rare event, but this possibility must not be ruled out (2). The nonspecific amplicons of some strains of *V. vulnificus* can be distinguished from the specific amplicon if the positive control is included in the gel electrophoresis analysis. Therefore, we do not think that it is necessary to run additional tests to exclude the possibility that the amplicon resulted from amplification of the *V. vulnificus* nucleotide sequence. However, if desired, such additional tests are available. This can be a simple biochemical test, for example, growth in 8% NaCl (3). Alternatively, the modification of the PCR protocol described above, in which the PCR primer set is substituted by the primer set 5′-AGCC CGCTTTCTTCAGACTC-3′ and 5′-AACGAGTCTTCTGC

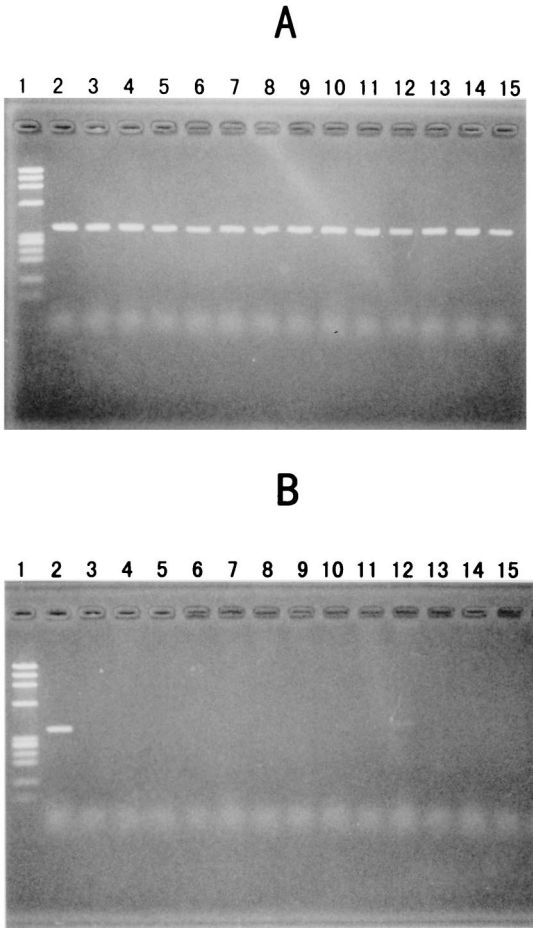

FIG. 2. PCR assay detects the *toxR* gene of *V. parahaemolyticus*. The reference strains are described in Table 3. (A) Reference strains of *V. parahaemolyticus*; lane 1, ϕX174 phage DNA digested with *Hae*III (molecular weight markers); lanes 2 to 15, reference strains 1 to 14 (Table 3), respectively. (B) Reference strains of non-*V. parahaemolyticus* species; lane 1, ϕX174 phage DNA digested with *Hae*III; lane 2, reference strain *V. parahaemolyticus* 13 (a positive control); lanes 3 to 16, reference strains 15 to 28 (non-*V. parahaemolyticus* species), respectively. (C) Comparison of selected strains of *V. parahaemolyticus* and all test strains of *V. vulnificus*; lane 1, ϕX174 phage DNA digested with *Hae*III; lanes 2 to 6, *V. parahaemolyticus* strains (positive controls); lanes 7 to 17, test strains of *V. vulnificus*.

ATGGTG-3′ or the primer set 5′-CGCTTTCTTCAGACTCA AGC-3′ and 5′-AACGAGTCTTCTGCATGGTG-3′, can be used; all strains of *V. parahaemolyticus* produced amplicons of 399 or 394 bp, respectively; none of the *V. vulnificus* strains produced any amplicons; some strains of *V. alginolyticus*, however, produced the amplicons (data not shown).

Growth of the test strain in LB broth at 37°C was used in the test described above. This growth condition was chosen for the organisms of clinical significance and is not suitable for most nonpathogenic *Vibrio* species in the marine environment. To confirm the specificity of the primer set, the standard strains belonging to 26 species of the genus *Vibrio* (those with specific strain numbers in Table 1) were also grown to good turbidity in Marine Broth 2216 (Difco Laboratories) at 25°C and were subjected to the PCR assay. All these strains gave negative results, whereas the *V. parahaemolyticus* strains grown under the same conditions gave positive results.

All 373 strains identified as *V. parahaemolyticus* carried the *toxR* gene, as described above. Of these strains, 47 strains showed atypical biochemical characteristics such as negative results by tests for lysine or ornithine decarboxylation or indole production or the Simmons citrate test and positive results by the rhamnose fermentation test (Table 2). To support the identification, these rare strains were examined for the presence of the *V. parahaemolyticus gyrB* gene by the PCR method. The PCR for the specific detection of the *V. parahaemolyticus gyrB* gene was carried out essentially as described by Venkateswaran et al. (20). The boiled culture supernatant was prepared as described above, diluted to 1:10, and used as the template solution. We used an annealing temperature of 60°C rather than 58°C, which was specified by Venkateswaran et al. (20), because in our hands *V. alginolyticus* strains gave the amplicons of the expected size at the latter annealing temperature. All strains tested gave positive results.

In conclusion, the PCR protocol for the *V. parahaemolyticus toxR* gene established in this study is specific and rapid (amplification can be achieved in 2 h). Simultaneous use of this PCR method and the PCR method for the detection of virulence genes like *tdh* and *trh* (17) would be useful for the rapid investigation of suspected *V. parahaemolyticus* strains isolated from clinical specimens and food samples implicated as sources of infection for patients with cases of food poisoning.

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